Journal of Medical Virology 53:4–12 (1997)

Effect of Herpes Simplex Virus on the DNA of Human Papillomavirus 18

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Some types of human papillomaviruses (HPV) appear to be associated with carcinoma of the cervix or other tissues, but patients infected with HPV do not necessarily develop carcinoma. Some epidemiological studies of risk factors for cervical carcinoma have indicated the involvement of herpes simplex virus (HSV). To study the effect of HSV on the genome of HPV, total DNAs were extracted and analyzed after HeLa cells, or A431 cells, transiently transfected with HPV18 DNA, were infected with HSV-1 or -2 for 24 hours. In HeLa cells, integrated HPV18 DNA was amplified almost threefold. In A431 cells, HPV 18 DNA fragments, sensitive to the restriction enzyme Mbo I, indicated newly replicated DNA. Replication intermediates were detected when the DNA was resolved by two-dimensional gel electrophoresis. This study showed that HSV caused some amplification of HPV and indicated the possibility of HSV involved in the integration and amplification of HPV in host cells. ***J. Med. Virol. 53:4–12, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: human papillomavirus; herpes simplex virus; replication; integration

INTRODUCTION

Human papillomavirus (HPV) is classified into papillomavirinae of papovaviridae and possesses a genome of closed circular DNA of about eight thousand nucleotides [Howley, 1996; Shah and Howley, 1996]. Open reading frames are located on one strand containing an early region, late region, and long control region (LCR). Open reading frames in the early or late region are described as E or L, followed by numerals. The functions of early genes are initiation of DNA replication (E1), transcriptional regulation/DNA replication (E2), binding to p53 (E6), and binding to pRB (E7). The functions of binding to p53 or pRB are characteristic to malignant types of HPV such as HPV16 or HPV18. HPV16 E5 genes were reported to increase the proliferative activity of human keratinocytes [Storey et al., 1992], but the E5 gene is not expressed in most HPV-positive cancers. Late genes are composed of two genes, L1 and L2. The former is a major capsid protein and the latter is a minor capsid protein. HPV infects a germ cell of the basal cell layer of squamous epithelium and begins to replicate using the replication machinery of the host cell [Howley, 1996]. This replication is initiated from the recognition of the replication origin of HPV by a complex consisting of E1/E2 and cellular DNA polymerases [Park et al., 1994], then proceeds bidirectionally with theta-form intermediates [Auborn et al., 1994]. As the basal cell layer differentiates to the granular layer, the genome of HPV replicates vegetatively and is packaged with late gene products to produce infectious particles. Clinically, this stage persists asymptomatically or as a benign lesion. On the other hand, it is well known that HPV genomes are mostly integrated in cervical carcinoma cells [Cullen et al., 1991], often as concatemers [Schwartz et al., 1985]. The integrated concatemers are amplified further with cellular flanking sequences [Wagatsuma et al., 1989]. The integration disrupts the function of E2, resulting in the loss of repression of promoters in the LCR and the constitutive expression of E6/E7 genes. Furthermore, the integration of HPV DNA into the human genome leads to increased stability of E6 and E7 mRNAs [Jeon and Lambert, 1995]. The products of E6/E7 genes of malignant types of HPVs interact with tumor suppressor proteins p53 [Scheffner et al., 1990] and pRB [Heck et al., 1992].

HPVs cause carcinomas, especially of the cervix, upper respiratory tract, and mouth to esophagus. HPVs are detected in more than 90% of cervical carcinomas. There is a regional difference in incidence of HPV type, but HPV16 is more common than HPV18. HPV18 tends to be present in undifferentiated and adenosquamous carcinoma of the cervix, whereas HPV16 is not found in these types of carcinomas but tends to be present in differentiated or large-cell types of carcinoma. Carcinomas associated with HPV18 may progress more rapidly than those associated with HPV16.

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Accepted 11 March 1997

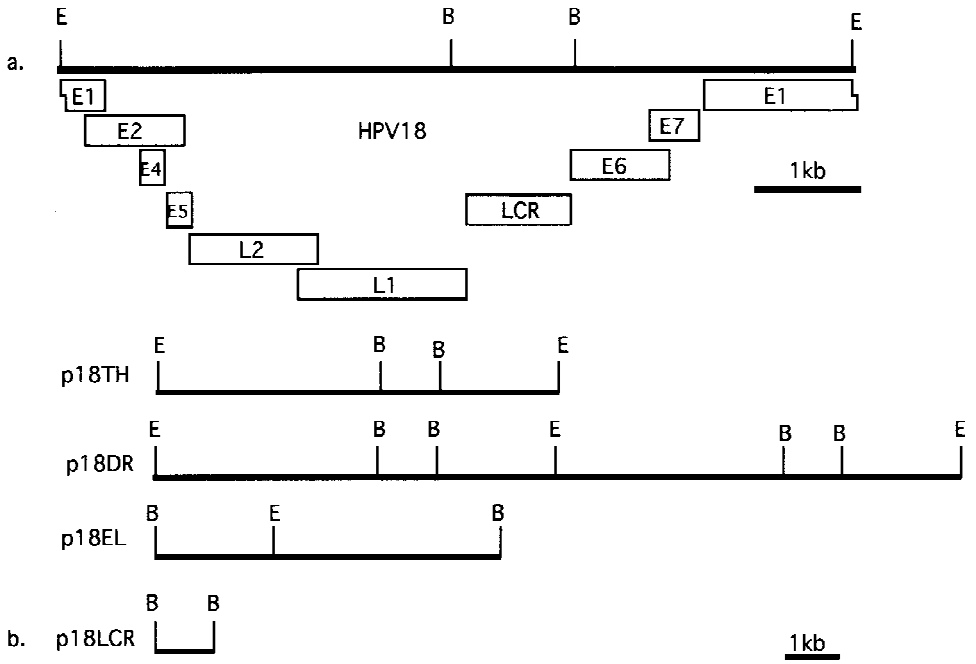

Fig. 1. Structure of HPV18 genome and its constructs. Eco RI fragment of HPV18 is shown in **a.** Each genome region is shown in open boxes. E1 ORF is cut by Eco RI at nucleotide position 2440. The constructs used in the experiments described in the legend to Figure 4 are shown in **b.** Plasmid p18TH was a construct of the whole genome of HPV18 inserted into pGEM3z at the Eco RI site. Plasmid p18DR is a construct of a head-to-tail dimer of HPV18 Eco RI fragments. p18LCR is the construct of a Bam HI fragment (1047bp) containingthe LCR region. Plasmid p18EL is a construct of a Bam HI fragment (6810bp) containing the early-to-late region. All these constructs contain pGEM3z as a vector. E and B represent the restriction enzymes Eco RI and Bam HI, respectively. HPV18 and pGEM3z consist of 7857 and 2743 nucleotides, respectively.

Many epidemiological and experimental studies on the association of cervical carcinoma, HPV, and herpes viruses have been reported since HPVs were discovered in cervical carcinomas. The influence of gene expression of HPV by herpes viruses [Gius and Laimins, 1989] and the influence of gene structure of papovaviruses containing HPV by herpes viruses [zur Hausen, 1996] have been noted. We considered that HSV may act on the structure of the HPV genome and break the natural life cycle of HPV as an episome, and thus lead to integration and amplification of integrated HPV DNA. Replication of integrated and episomal HPV18 DNA with HSV infection is described, although small in quantity but specific.

MATERIALS AND METHODS

Cell Lines and Viruses

A431 was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and maintained with Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum. The cell line was established from human epidermoid carcinoma [Giard et al., 1973] and collected by American Type Culture Collection. We confirmed that A431 cells did not contain the HPV18 sequence by Southern hybridization in the condition described below. HSV-HF, as a representative of HSV type 1, was a gift from Dr. S. Nii. HSV-UW268, as a representative of HSV type 2, was donated by Dr. K. Hayashi [Wentworth and French, 1969].

Plasmids

The plasmid, pHPV18, in which the Eco RI fragment of HPV18 was cloned, was a kind gift from Dr. H. zur Hausen [Boshart et al., 1984]. Vector pBR322 of pHPV18 was replaced with pGEM3z (p18TH). To reconstruct the Eco RI site in the E1 gene, the Eco RI fragment of HPV18 was ligated, then partially digested with Eco RI. A head-to-tail dimer was cloned from this partially digested Eco RI fragment (p18DR) to form p18LCR and p18EL. HPV 18 was cut with Bam HI at positions 119 and 6929. The nucleotide position 6929 to 119 encoding the long control region (LCR) was ligated to pGEM3z (p18LCR). The nucleotide position 119 to 6929 encoding early and late genes was ligated to pGEM3z (p18EL). These are shown in Figure 1.

Human insulin gene from nucleotide position -59 to 1547 inserted in pBR322 was donated by Dr. G.I. Bell [Bell et al., 1980].

Transfection

A431 cells were cultured to be 70–80% confluent in 60-mm dishes. After removing the medium, the cells were washed twice and covered with 3 ml of serum-free DME. Ten micrograms of test plasmid and 30 μg of Lipofectin® (GIBCO BRL) were mixed to form a complex. This mixture was added to the DME covering the washed cells. Fifteen hours later, an equal volume of DME containing 20% fetal bovine serum was added.

TABLE I. The Amplification of HPV18 DNA Integrated Into HeLa Cells by HSV Infection

	HSV-HF	HSV-UW268	Mock infection	HF/mock(%)	UW268/mock(%)	Mock/mock(%)
Probe/pHPV18	4129[a]	5109[a]	960.0[a]	430.1	532.2	100.0
Probe/hIns	3409[a]	4288[a]	2338[a]	145.8	183.4	100.0
HPV18/hIns(%)	121.1	119.2	41.06			
Amplification(%)	295.0	290.2	100.0			

[a]square pixels of shaded area of Figure 2c.

HSV Infection

After 24-hours of incubation, the transfected cells were infected with HSV at multiplicity of infection one, and 5 ml of DME containing 2% fetal bovine serum was added. Acyclovir, an inhibitor of HSV DNA polymerase, was added at 20 μg/ml just after the infection with HSV. The cytopathic effect of HSV was inhibited at this concentration.

DNA Isolation and Southern Blot Hybridization

The cells were digested with Proteinase K 24 hrs after HSV infection. After phenol extractions, the extracts were precipitated with ethanol and resuspended in TE (10mM Tris/HCl, pH 8.0, 1mM EDTA). Ten micrograms of extracted DNA was digested with six units of Dpn I or Mbo I for four hours. After electrophoresis, the gel was treated with HCl and blotted onto positively charged nylon membranes by absorbing the 0.4 M NaOH. DNA for a probe was labeled with ^{32}P by multi-primer extension (Amersham, Multiprime DNA labeling system). The membranes were pre-hybridized in 4 × SSPE (SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 2% SDS (sodium dodecyl sulfate), 0.5% skim milk, and 0.5 mg/ml of heat-denatured salmon DNA for 3 hours at 68°C, then hybridized in 4 × SSPE, 2% SDS, 0.5% skim milk, and the heat-denatured probe of 10^7 cpm (10^9 cpm/mg of DNA) for 15 hours at 68°C. The membranes were successively washed with 2 × SSC containing 0.1% SDS and 0.1 × SSC containing 0.1% SDS at room temperature, respectively, for 15 minutes, 0.1 × SSC containing 0.1% SDS at 68°C for 60 minutes, and 0.1 × SSC containing 0.1% SDS at room temperature briefly. Membranes were exposed to X-ray film (Fuji RX) at −70°C. For reprobing, the membrane was boiled for 15 minutes twice with 0.1 × SSC and washed at room temperature, then confirmed by exposing to X-ray film.

Two-Dimensional Gel Electrophoresis

To identify the replication intermediate by the two-dimensional agarose gel electrophoresis [Brewer and Fangman, 1987], the DNA digested with Eco RI and the molecular weight markers were first electrophoresed through 0.4% agarose in 0.5 × TBE without ethidium bromide at 1 volt/cm at room temperature. Each lane of the sample or marker was excised respectively after the first electrophoresis. The lane containing the marker was stained with ethidium bromide and photographed with a scale. The lane of the sample was rotated by 90°, placed on the top of a gel tray, and fixed with 1.0% agarose gel containing 0.3 μg/ml of ethidium bromide and 0.5 × TBE. The second electrophoresis was run at 7 volts/cm at 4°C.

Densitometric Evaluation of Hybridization Signals

Autoradiograms of Southern hybridization were scanned using a personal computer (Macintosh) with a gray scale of 256 gradations, using a trans-illuminator. Contrast enhancement and density profiling of these images were achieved using the NIH Image 1.55. The intensity of the hybridization signals was measured as the area representing HPV18 or human insulin by this software. To calculate the amplification ratio, the ratio of amount of HPV18 to insulin was first calculated in each lane containing HSV-HF, -UW268, or mock-infected cells, then the ratio in the lane of HSV-HF or -UW268 was divided by that of the mock infection.

RESULTS

HPV18 DNA Integrated in HeLa Cell Was Amplified Almost Threefold by HSV Infection

The influence of HSV on integrated HPV18 DNA of HeLa cells was examined. The results are shown in Table I. Autoradiograms and their graphs by NIH image are shown in Figure 2. The signals of the reprobed autoradiogram in Figure 2bi were so weak that the processed image is also shown in Figure 2bii. As shown in Table I, in the cells infected with HSV-HF or HSV-UW268 for 24 hours, HPV18 was amplified threefold. This calculation was based on the assumption that human insulin gene was not amplified by HSV and corresponded to the amount of total cellular DNA. Human placental DNA used as a control indicated that cross-hybridization by HPV18 probe was excluded under the hybridization conditions.

Episomal HPV18 DNA Was Amplified by HSV Infection

In HeLa cells, HPV18 DNA is integrated as a concatemer, but HPV genomes exist as episomes during the natural replication cycle. To understand the effect of HSV on the natural replication cycle of HPV, we studied the influence of HSV on the episomal HPV DNA. The constructs of HPV18 DNA introduced into A431 cells were examined after infection with HSV-HF or HSV-UW268.

Cells were transfected with pHPV18 and infected with HSV-HF. To determine whether HPV18 DNA was synthesized in A431 cells, the extracted high-molecular

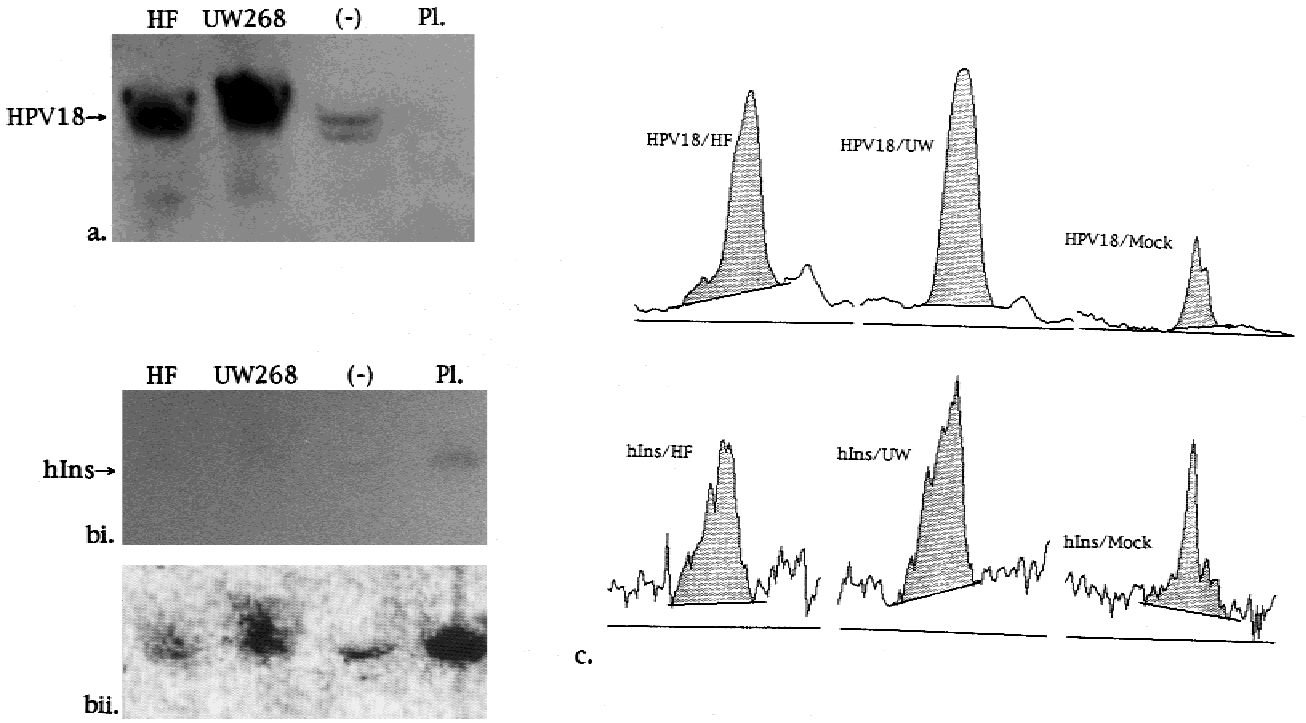

Fig. 2. The amplification of HPV18 integrated into HeLa cells by HSV-1 or -2. **a:** Southern hybridization of HeLa cell infected with HSV. Ten micrograms of each total DNA extracted from HeLa cells after HSV or mock infection and a human placenta was digested with Eco RI. The blot was hybridized with HPV18 Eco RI fragment as a probe. HF: HeLa cell DNA infected with HSV-HF. UW: HeLa cell DNA infected with HSV-UW268. (−): Mock infection. P1: Human placental DNA. **b:** Reprobing with human insulin gene to control the total amount of DNA. The washed membrane was reprobed with the human insulin gene to control the quantity of HeLa cell DNA **(bi).** The contrast of 2bi was enhanced by NIH Image **(bii). c:** Scanned graphs of autoradiograms. The autoradiograms of 2a and 2bii were scanned. Calculated areas are indicated as shadows. The left side of each graph corresponds to the top of autoradiograms, and the right side of each graph corresponds to the bottom of autoradiograms. HPV18/HF, HPV18/UW268, and HPV18/mock are scanned graphs of the lane HF, UW268, and (−) of 2a, respectively. hIns/HF, hIns/UW268, and hIns/mock are scanned graphs of the lane HF, UW268, and (−) of 2bii, respectively.

weight DNAs were digested with the restriction enzymes Dpn I or Mbo I, which recognize the same site. Dpn I cuts only dam-methylated DNA, which is synthesized in *E. coli*, whereas Mbo I does not strictly cut dam-methylated DNA. In contrast, Mbo I cuts the DNA replicated in eukaryotic cells. We confirmed that Mbo I did not digest the constructs used for transfection. Digested DNAs were separated by electrophoresis in 1.2% agarose gels containing 0.5 × TBE. The results of the Southern hybridization are shown in Figure 3. Fragments produced by Dpn I that were indicated as Dpn I^r were weak but distinct and larger than those from *E. coli* (lane 2). In addition, fragments resulting from Mbo I digestion coincided with those from *E. coli* (lane 3). Such fragments were absent in the lanes containing DNAs exposed to acyclovir, after digestion with Dpn I or Mbo I. In the presence of phosphonoacetate, an inhibitor of HSV DNA polymerase, or after mock infection, the findings were the same as those with acyclovir (data not shown).

These findings suggested that the HPV18 DNA introduced into A431 cells replicated with the aid of the DNA polymerases of HSV-HF, though the level of replication was low.

Cis Element of HPV18 Used for Replication by HSV Was Not Identified

To identify the genomic region of HPV 18 involved in the replication by HSV, the constructs shown in Figure 1 were prepared. A431 cells were transfected with each of these constructs, then infected with HSV-HF or HSV-UW268. Total DNAs were digested with Mbo I or Dpn I and resolved by agarose gel electrophoresis. The molecular marker of p18TH digested with Dpn I was not used to avoid the contamination to the neighboring lanes.

Fragments generated by Mbo I digestion of p18TH infected with HSV-UW268 (Fig. 4, lane 2) were detected, but fragments resistant to Dpn I were not evident. The reason for this may be that the fragments were not separated sufficiently or were too large to be transferred efficiently. Nevertheless, the presence of fragments generated by Mbo I digestion indicates the de novo synthesis of DNA. It is difficult, however, to identify the region containing the origin of replication of HPV18 by HSV because we could not detect clear fragments generated by Mbo I digestion in the lanes containing p18LCR or p18EL, even though the transfection and infection experiments were repeated sev-

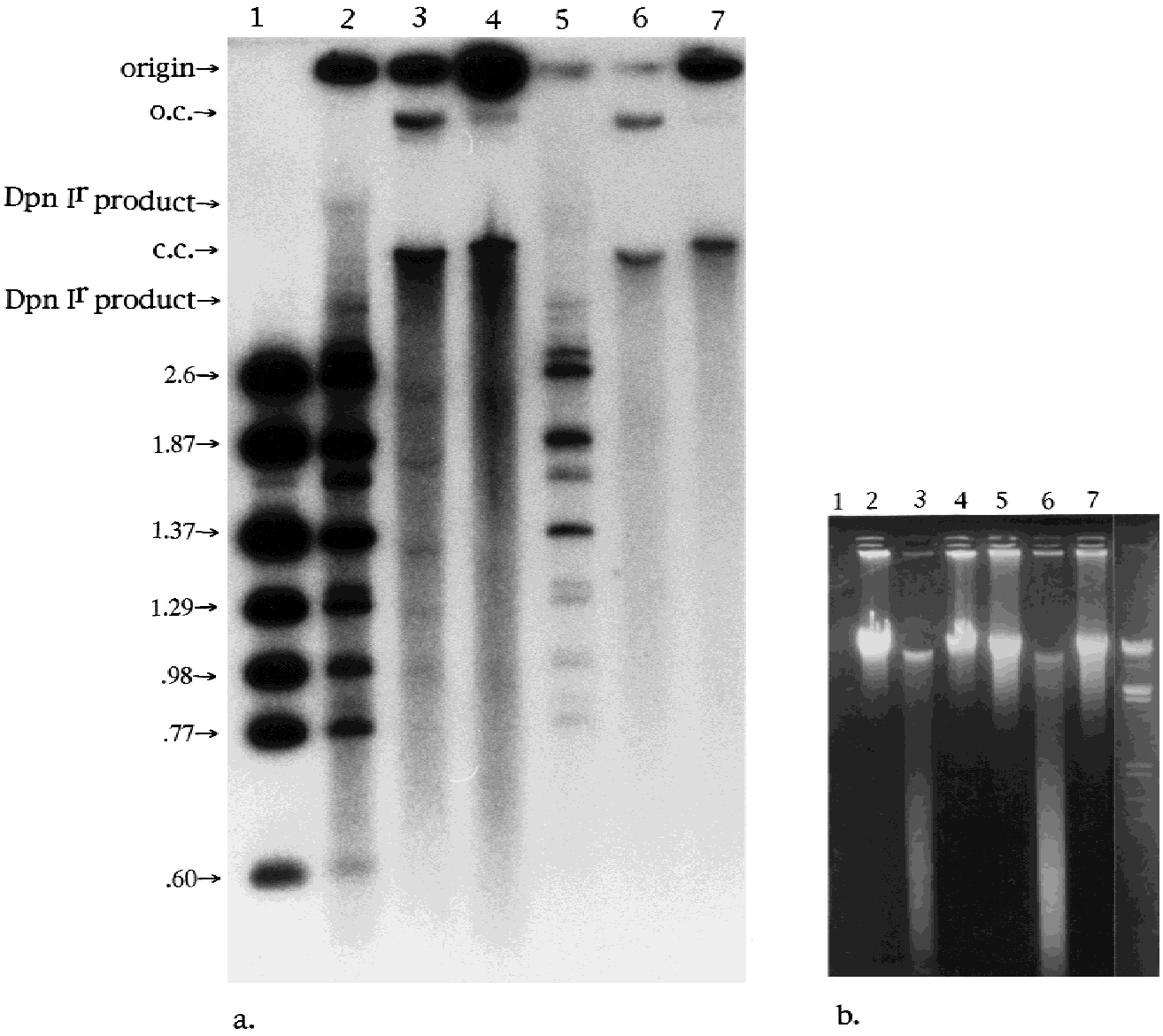

Fig. 3. The amplification of pHPV18 by HSV-HF. **Lane 1**, pHPV18 digested with Dpn I as a marker; **lane 2**, DNA was extracted from A431 cells transfected with pHPV18, then infected with HSV-HF, and digested with Dpn I; **lane 3**, same DNA as lane 2, but digested with Mbo I; **lane 4**, same DNA as lane 2, without digestion. **Lanes 5, 6**, and **7**, same as lanes 2, 3, and 4, but the A431 cells were exposed to 20 mg/ml of acyclovir. Molecular mass is shown in kb at the left of the autoradiogram; origin, the origin of electrophoresis; o.c., the nicked, relaxed molecule, form II; c.c., unnicked molecule, form I. **a:** The Southern hybridization with pHPV18 cut with Eco RI as a probe. **b:** The photograph of agarose gel electrophoresis after DNA digestions. The right-end lane is HindIII and Eco RI-digested γ DNA.

eral times. Thus, attempts were made to identify the origin of replication and the replication intermediate by means of two-dimensional agarose gel electrophoresis.

Replication of HPV18 Construct by HSV Starts From Multiple Sites

Two-dimensional agarose gel electrophoresis is based upon the discrepancy between mobility in the gel and the size of the DNA due to its conformation. The open circle and fork configuration of DNA migrate more slowly than the linear configuration at the same molecular weight. This anomalous electrophoresis is exaggerated by increasing voltage, agarose concentration, and ethidium bromide. The first electrophoresis is proportional to the molecular weight, and the second electrophoresis exaggerates anomalous mobility as much as possible. The total DNA in cells transfected with p18TH and infected with HSV-UW268 presented in lane 2 of Figure 4 was cut completely with Eco RI to divide p18TH into the HPV18 Eco RI fragment and pGEM3z. An autoradiogram of this two-dimensional agarose gel electrophoresis is shown in Figure 5a. A

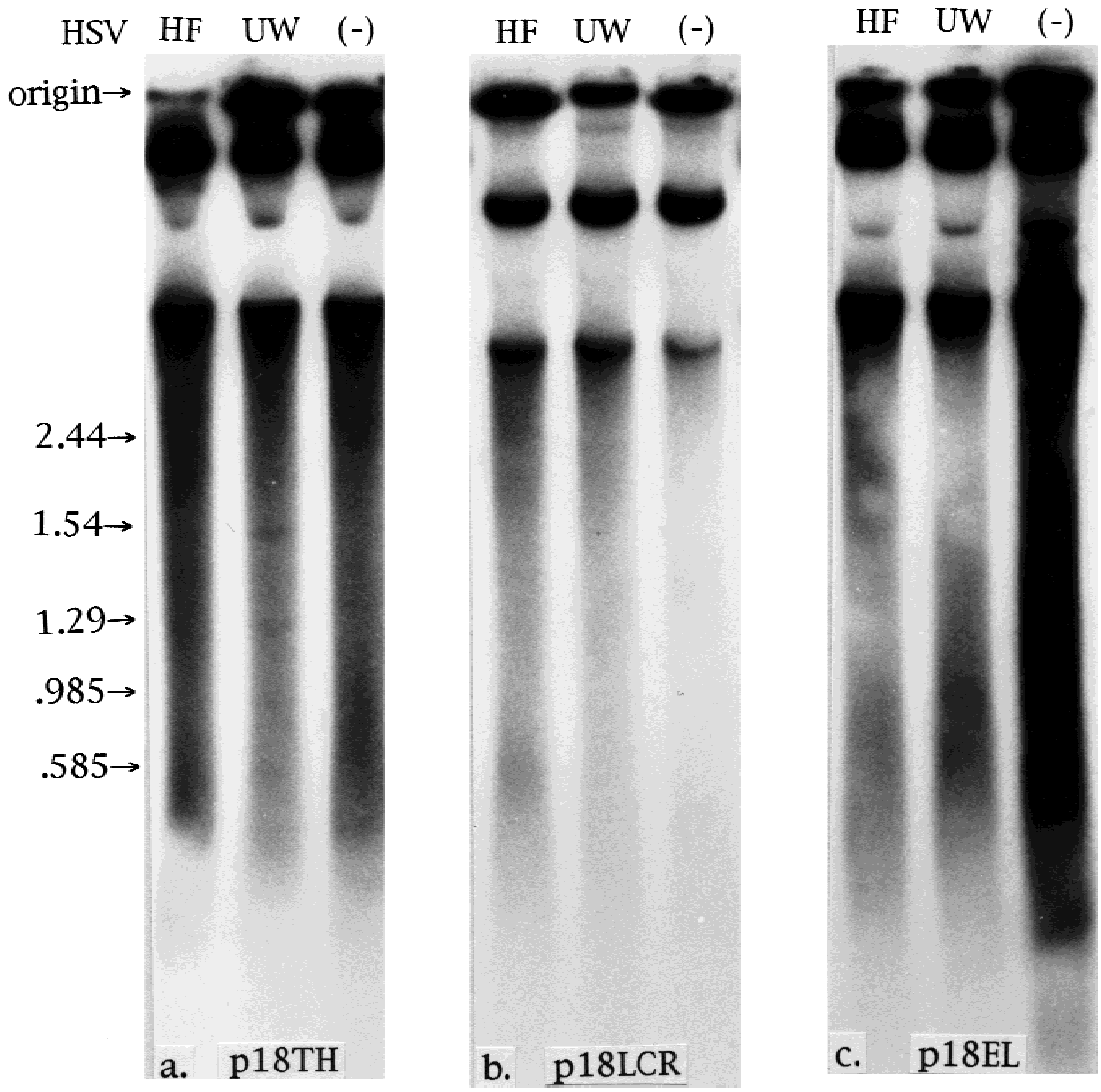

Fig. 4. Mbo I analysis of the transfected HPV18 DNA after HSV infection. A431 cells were transfected with each construct, then infected with HSV for 24 hours. High-molecular weight DNAs were digested with Mbo I, separated by electrophoresis in a 1.2% agarose gel, then Southern hybridized with p18TH cut with Eco RI as a probe. DNAs transfected with p18TH, p18LCR, and p18EL following digestion with Mbo I are shown in **a, b,** and **c,** respectively. In each column, HF, UW, and (−) represent HSV-HF, HSV-UW268, and mock infection, respectively. The molecular mass is shown in kb on the left of the autoradiogram. Large DNA bands from the top represent the origin of electrophoresis, form II, form III, and form I, respectively.

sketch is also shown in Figure 5b. To understand the autoradiogram, the model of analysis by this method is shown in Figure 5c [Brewer and Fangman, 1987]. In contrast to the thick arc representing simple linear DNA (α), the arcs with no continuity were thought to be intermediates of replication. The arc (β) toward the upper left might correspond to the double-Y configuration and suggests that the replication of this molecule originated from vector pGEM3z. The upward convex arc (γ) might correspond to the simple-Y configuration. The large upward convex arc (δ) might correspond to the bubble configuration, and showed that the replication of this molecule originated within the HPV 18 Eco RI fragment.

DISCUSSION

It was shown that the integrated and episomal DNA of HPV18 introduced into cells could be amplified by HSV infection. During the period from infection with HPV to the development of carcinoma, there is a marked difference in the existence mode of the HPV genome between benign and malignant lesion. HPV replicates as autonomously replicating episomes with a combination of helicase coded by HPV and DNA polymerases of cells in benign lesions and produces progeny, whereas it is often integrated as a concatemer and does not produce progeny in malignant lesions. Only a minority of patients infected with HPV develop the car-

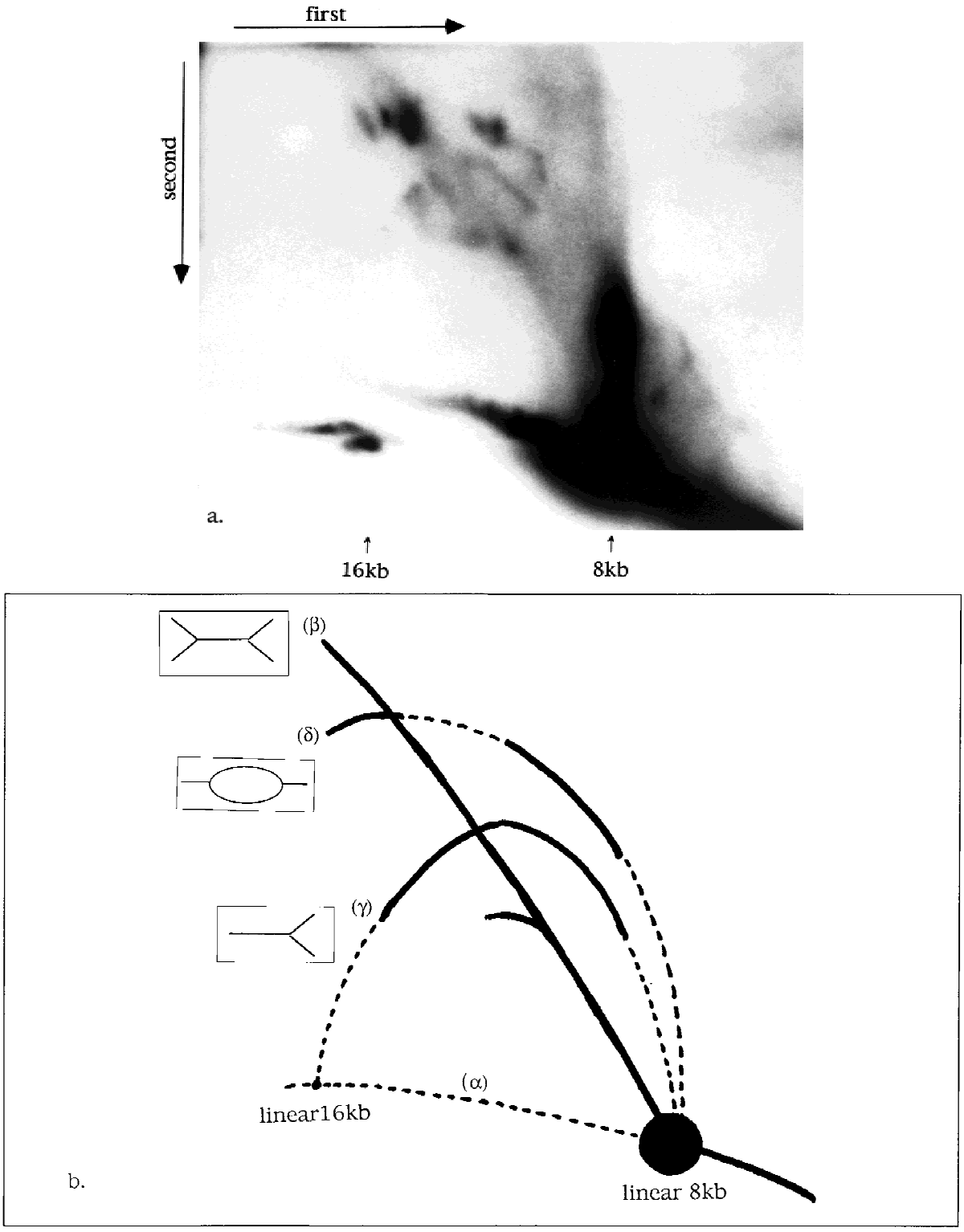

Fig. 5. Two-dimensional agarose gel electrophoresis showing replication intermediates of HPV18 DNA replicated by HSV-2. **a:** Southern hybridization with HPV18 Eco RI fragment as a probe. The DNA shown in lane UW of Figure 4 column **a** was digested with Eco RI. The first dimension consisted of 0.4% agarose in 0.5 × TBE without ethidium bromide at 1 volt/cm. The second dimension consisted of 1.0% agarose gel containing 0.3 μg/ml of ethidium bromide and 0.5 × TBE at 7 volts/cm at 4°C. The direction of electrophoresis is shown as an arrow. The site for the linear monomer (8kb) of p18TH or the linear dimer (16kb) at the first electrophoresis is shown with arrows below the autoradiogram. **b:** A sketch of the autoradiogram. The arcs α, β, γ, and δ are described in the text. Putative signals were described with dotted line.

cinoma, and it takes a long time to develop it, suggesting that something other than HPV must be involved in developing carcinoma with HPV [DiPaolo, 1993; Chen, 1994]. Since some papillomaviruses other than HPV cause carcinoma with cocarcinogen, it is natural to think that HPV causes carcinoma with unidentified cocarcinogen. A few epidemiological findings support this [Hildesheim et al., 1991]. They reported that the risk factor of cervical carcinoma in the case positive both for HPV and HSV is higher by twice than the case positive only for HPV. If HSV plays a role in carcinogenesis by HPV, it may act on the structure because carcinoma never reverts to a benign state after HSV disappears. The findings obtained in this study are limited because of the small amount of DNA amplified by HSV. However, the DNA amplified by HSV was thought to be replicated following the rolling circle mechanism characteristic of HSV DNA polymerases [Danovich and Frenkel, 1988; Skaliter and Lehman, 1994]. In this study, we could not show that the newly amplified DNA by HSV was a concatemer, but the simple-Y configuration shown in Fig. 5. may correspond to rolling circle replication. This notion coincides with the presence of an oligomer or concatemer in carcinoma cells [Boshart et al., 1984; Choo et al., 1989; Pfister et al., 1983; Yutsudo et al., 1985]. Furthermore, the amplification of integrated HPV18 DNA in HeLa cells may coincide with the HPV 16 DNA amplification with flanking cellular sequences [Wagatsuma et al., 1990].

In experiments described above, the replication of constructs of HPV18 originated from multiple sites, including those of a vector. There is no homology of replication origins between HPV18 and HSV. Even a plasmid, pUC18, is replicated efficiently by HSV-1 replication proteins without the origin-binding protein in vitro [Skaliter and Lehman, 1994]. The DNA in in vitro system does not exist in a complex with histones and other proteins, whereas the DNA in eukaryotic cells exists in chromatin-like structures [Givens et al., 1996]. It is possible that the replication proteins of HSV, except for the origin-binding protein, do not select the sequence, but attach to loose sites on double strands, where it is unwound, and replicate DNA from there. Papillomavirus DNA replicated by HSV would not be packaged into virions because it would be a linear concatemer. It would then be degraded or integrated into the host genome, as if linear molecule of HPV DNA is transfected artificially. As a result of the destruction of E2 repressor gene, a cell expressing E6/E7 genes constitutively would gain immortality, then progress into carcinoma following accumulation of mutation.

On the assumption that HSV replicates by the rolling circle mechanism recurrently and the cells do not die, we can explain the HPV DNA integration, amplification, and amplification involving the flanking host cell DNA [Wagatsuma et al., 1990] in cervical carcinoma cells. There is a counter-argument that cells infected with HSV not in latent form must die and do not progress into carcinoma. HSV is excreted frequently and asymptomatically from the external genitalia of women infected with HSV type 2 [Brock et al., 1990]. A set of six genes of HSV DNA replication, except for the HSV origin-binding protein (UL9), can amplify integrated SV40 DNA [Heilbronn and zur Hausen, 1989]. These proteins replicate the DNA by the rolling circle mechanism but do not kill the cells. Some mutant viruses or unknown mechanisms that do not kill the cells and express replication proteins must be considered. Since *herpes viridae* have the common property of rolling circle replication, herpes viruses other than HSV can possibly replicate HPV as HSV [Heilbronn et al., 1993]. The notion that the HPV DNA replicated by HSV is prone to integrate into the host genome must be confirmed experimentally.

ACKNOWLEDGMENTS

We thank Dr. Haruo Ohmori at the Institute of Virology, Kyoto University, and Drs. Masuo Yutsudo and Hideo Shinagawa at the Institute for Microbial Disease, Osaka University, for their helpful discussion. This work was supported in part by a grant from the Creative Research Funds of Osaka Prefecture.

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